The PDK1 master kinase is over-expressed in acute myeloid leukemia and promotes PKC-mediated survival of leukemic blasts

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The PDK1 master kinase is over-expressed in acute myeloid leukemia and promotes PKC-mediated survival of leukemic blasts

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ABSTRACT

PDK1 is a master kinase that activates at least six protein kinase groups including AKT, PKC and S6K and is a potential target in the treatment of a range of malignancies. Here we show overexpression of PDK1 in over 40% of myelomonocytic acute leukemia patients. Overexpression of PDK1 occurred uniformly throughout the leukemic population, including putative leukemia-initiating cells. Clinical outcome analysis revealed PDK1 over-expression was associated with poorer treatment outcome. Primary acute myeloid leukemia blasts over-expressing PDK1 showed improved *in vitro* survival and ectopic expression of PDK1 promoted the survival of myeloid cell lines. Analysis of PDK1 target kinases revealed that PDK1 overexpression was most closely associated with increased phosphorylation of PKC isoenzymes and inhibition of PKC strongly inhibited the survival advantage of PDK1 over-expressing cells. Membrane localization studies implicated PKCα as a major target for PDK1 in this disease. PDK1 over-expressing blasts showed differential sensitivity to PDK1 inhibition (in the low micromolar range) suggesting oncogene addiction, whilst normal bone marrow progenitors were refractory to PDK1 inhibition at effective inhibitor concentrations. PDK1 inhibition also targeted subpopulations of leukemic blasts with a putative leukemia-initiating cell phenotype. Together these data show that overexpression of PDK1 is common in acute myelomonocytic leukemia and is associated with poorer treatment outcome, probably arising from the cytoprotective function of PDK1. We also show that therapeutic targeting of PDK1 has the potential to be both an effective and selective treatment for these patients, and is also compatible with current treatment regimes.

Introduction

Advances in the understanding of the complex and heterogeneous molecular mechanisms underlying acute myeloid leukemia (AML) have fuelled a drive towards targeted therapy. The development of novel agents targeting individual molecular lesions, used either alone, in combination, or as an adjunct to conventional chemotherapy holds considerable promise for improving clinical responses without increasing treatment-related toxicity.[1]

Phosphoinositide-dependent kinase (PDK1) is a serine/threonine protein kinase that phosphorylates and activates at least six kinase groups in the AGC superfamily.[2,3] Many of these kinases have been shown to be constitutively active in tumor tissue including: AKT,[4] S6K,[5] SGK,[6] RSK[7] and PKC isoforms.[8] Genetic knockout studies have demonstrated that PDK1 is essential for the activation of these kinases.[9,10] PDK1 is a constitutively active kinase[11] with substrate phosphorylation being largely regulated by co-localization or substrate conformation.[12] In the case of AKT, phosphorylation by PDK1 is dependent on phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) production that binds the pleckstrin homology domains of PDK1 and AKT and co-localizes these kinases at the plasma membrane. In contrast, phosphorylation of S6K, SGK and RSK by PDK1 is dependent on a conformational change in these kinases induced by cell stimulation. PKC isoforms are thought to be constitutively phosphorylated by PDK1 during synthesis and are vital for maintaining the stability of conventional and novel PKC isoforms.[10,13]

The ability of PDK1 to activate multiple substrates may explain the influence of this kinase on a variety of cellular processes including proliferation,[14] migration[15] and survival.[16] Constitutive knockout of PDK1 gives rise to embryonic lethality, but in contrast, hypomorphic mice (which express approx. 10% of the normal levels of PDK1) are viable and fertile suggesting that normal cells are able to compensate for low levels of PDK1 activity.[17] These mice are also resistant to hematologic malignancy as well as other cancers when crossed with the highly cancer-prone PTEN-deficient mice.[18] PDK1($^{-/-}$) ES cells also have low tumorigenic potential compared to PDK1($^{+/+}$)cells.[19] Consistent with these observations, PDK1 overexpression is a common feature of a wide variety of cancers[20-23] and an RNAi screen has identified PDK1 as being the most important factor in mediating resistance to tamoxifen in breast cancer.[24] An essential role for PDK1 has also recently been identified in pancreatic cancer.[25] These data, together with its role as a master kinase regulator, have established PDK1 as a significant drug target in cancer, and it is also one of the few kinases represented in higher eukaryotic genomes as a single isoform increasing its tractability as a drug target. Further, since 50% of all cancers including leukemias possess mutations in genes that dysregulate PIP3 production,[4,26] overstimulation of PDK1 signaling may be extremely common. At the same time, the fact that normal

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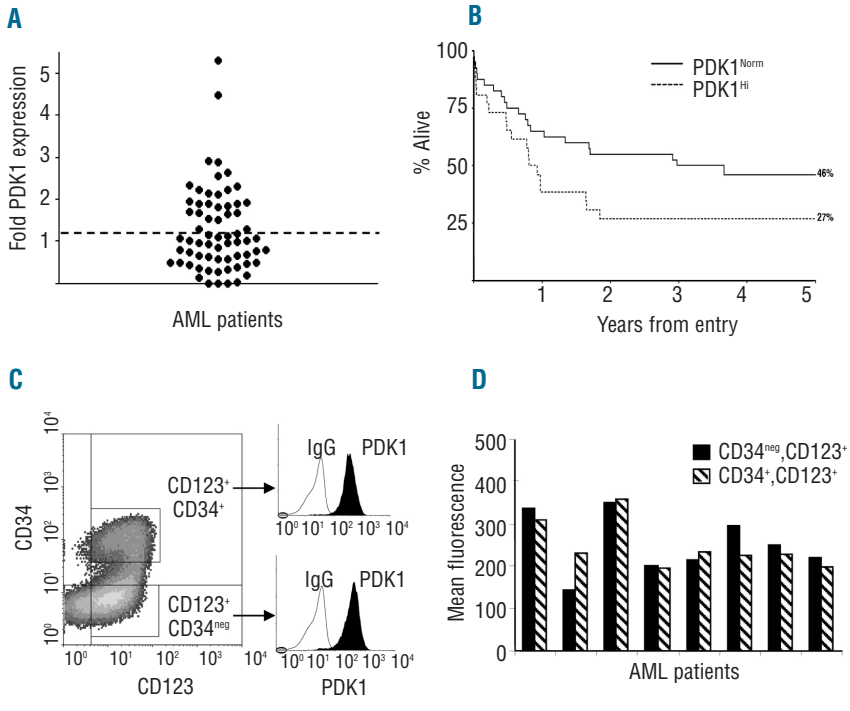

Figure 1. PDK1 overexpression confers poor clinical outcome in M4/M5 patients and is represented throughout the leukemic clone. (A) PDK1 protein expression in 66 myelomonocytic AML patients (data are expressed as fold change relative to normal bone marrow $CD34^+$ cells; n=5). The threshold of overexpression (mean+2SD) is indicated by the dotted line. (B) Kaplan-Meier plot for intensively treated patients significantly over-expressing PDK1 ($PDK1^{Hi}$) compared with those expressing statistically normal levels ($PDK1^{Norm}$) in M4/M5 AML (n=66). Hazard ratio 95% CI (0.98-4.21) *P*=0.05 adjusted for known prognostic variables. No significant difference in complete remission was observed. (C) Representative cytometric data demonstrating homogeneous intracellular PDK1 expression in leukemic ($CD123^+$) subpopulations. (D) Bar chart summarizing PDK1 expression in $CD34^+$ compared with $CD34^{neg}$ leukemic populations (n=8).

cells tolerate very low levels of PDK1 activity (similar to that which would be achieved through the use of a powerful PDK1 inhibitor[18,26]) suggests that PDK1 inhibition should be selectively toxic for cancer cells.

Previously we identified PDK1 overexpression in myelomonocytic AML (FAB M4 and M5).[27] Here we show that PDK1 overexpression is associated with poor clinical outcome, and that while overexpression promotes the survival of AML blasts, they are highly sensitive to PDK1 inhibition.

Methods

Cell culture and patients' samples

AML patient material (*Online Supplementary Table S1*) and normal bone marrow were collected with approval from the Research Ethics Committee for South East Wales and the Multi-Centre Research Ethics Committee for Wales in accordance with the Declaration of Helsinki. Mononuclear cells from AML patients were purified by Ficoll density gradient and analyzed for blast content by flow cytometry following staining with CD45. Samples with more than 70% blasts following density gradient fractionation were employed in subsequent analysis. AML blasts were cultured in IMDM (Sigma, Poole, UK) with 10% FCS (Biosera, Ringmer, UK). Normal bone marrow CD34+ cells were isolated using MiniMACS (Miltenyi Biotec, Bisley, UK) according to the manufacturer's instructions. Cell lines were cultured as recommended by ATCC-LGC (*http://www.lgcstandards-atcc.org*). All cultures were incubated at 37°C, 5% CO_2. Cell viability was determined by Trypan blue exclusion using a Vicell-XR viability analyzer (Beckman Coulter, Fullerton, CA, USA). PDK1 was subcloned into bicistronic PINCO vector and retrovirus generated as previously described.[27] Transduction efficiency of 40-50% was monitored through co-expression of GFP and positive cells selected using FACSAria cell sorter (BD Biosciences, Oxford, UK).

Western blot analysis

Sample preparation of fractionated and non-fractionated lysates and Western blotting was performed as previously described.[27,28] Further details are provided in the *Online Supplementary Methods.*

Flow cytometric analysis of cell survival and intracellular PDK1 staining

Cell survival/drug response assays were set up in 96 U-well plates with $5x10^4$ AML cells per well. Some experiments employed the PDK1 inhibitor, BX-795 (MRC Protein Phosphorylation Unit, University of Dundee, UK). For cytosine arabinoside (AraC) synergy experiments, dose response assays were set up for singly- and combination-treated AML cells using clinically relevant AraC doses. Cells were harvested after 48 h and resuspended in 1 μg/mL 7-amino-actinomycin D (7AAD) to determine viable cells remaining. In some experiments, samples were pre-stained with surface markers: CD34-FITC, CD123-PE and CD45-APC or isotype control (BD Biosciences, Oxford, UK), prior to harvesting and analysis on a FACSCalibur® cytometer (BD). EC50 and combination index values were determined using Calcusyn v.2.0 software (Biosoft, Cambridge, UK). For PKC inhibitor analysis, cells were treated with Chelerythrine (CC) and Bisindolylmaleimide (BIM1) (both LC laboratories) under reduced serum conditions and cell proliferation was measured by CellTiter96® MTS reagent (Promega).

For intracellular PDK1 staining, AML blasts were pre-stained for 30 min at 4°C with surface markers as above then fixed in fresh 2% paraformaldehyde for 20 min and permeabilized in PBS 0.1% Triton for 5 min before incubation with 1 μg/mL rabbit anti-PDK1 (1624-1; Epitomics, Burlingame, CA, USA) or rabbit IgG control for 30 min at 20°C. Finally, cells were labeled with goat-anti-rabbit PE-conjugated secondary antibody (L43004, Caltag Laboratories, Bucks, UK) for 30 min at 20°C.

Statistical analysis

Multivariable analyses were performed using either logistical or

Cox proportional hazard regression methods adjusted for age, white blood cell count, cytogenetic group, performance status, *de novo*/secondary disease and gender. Hazard ratios (HR) quoted for 95% confidence intervals. *P* values are two-tailed. For all other results, differences between mean values were compared by Minitab v.13 (Minitab Inc.; PA, USA) using Mann Whitney-U or paired t-test.

Results

PDK1 overexpression is associated with poor clinical outcome and occurs throughout the leukemic clone

To investigate the clinical significance of PDK1 overexpression in myelomonocytic AML patients, we analyzed PDK1 protein expression in a cohort of 66 patients (*Online Supplementary Table S1*) by Western blot analysis. We observed up to 5-fold overexpression in 42% of these patients (Figure 1A). Analysis of the association between PDK1 expression level, patients' characteristics and clinical outcome showed that overexpression was associated with poorer overall survival (Figure 1B).

AML is a clonally heterogeneous disease[29] where some abnormalities (such as FLT3) may occur only in a subpopulation of leukemic blasts; in addition there is functional heterogeneity defined by xenograft models where leukemia-initiating cells (LIC) are often (though not exclusively) associated with CD34 expression.[30] In order to determine whether PDK1 was over-expressed throughout the malignant clone, we determined PDK1 expression at a single cell level using flow cytometry. Intracellular staining of PDK1 was validated using KG-1 cells retrovirally transduced to over-express PDK1 (*Online Supplementary Figure S1*). We applied this method to analyze the expression of PDK1 in AML blasts that were surface-labeled with CD34, CD45 and the leukemic blast cell marker, CD123.[31] We analyzed 8 AML patients and in all cases found PDK1 to be uniformly expressed throughout the malignant clone (Figure 1C). Comparison of expression in $CD34^{neg}$, $CD123^{+}$ cells with the $CD34^{+}$, $CD123^{+}$ subset of blasts showed that PDK1 was expressed at similar levels in both populations in all patients tested (Figure 1C and D). These data show that PDK1 overexpression is an inherent property of the entire leukemic blast population including elements most closely associated with the LIC population.

Together these data indicate that PDK1 overexpression is a common abnormality in myelomonocytic AML, occurs throughout the leukemic clone, and negatively impacts on the survival of AML these patients.

PDK1 overexpression promotes cell survival in myelomonocytic AML

Since PDK1 targets a number of AGC kinases that are associated with promoting cell survival,[3] we examined whether PDK1 overexpression promoted the survival of myelomonocytic AML blasts. To address this, we compared the *in vitro* survival of over-expressing ($PDK1^{Hi}$) blasts compared with the $PDK1^{Norm}$ control group in growth factor-free cultures for 48 h by Trypan blue exclusion. Those patients with high PDK1 expression displayed significantly better survival (82%±13.3 in $PDK1^{Hi}$ *vs.* 57%±14.9 in $PDK1^{Norm}$; $P<0.001$) (Figure 2A). Even when assayed in the presence of growth factors (IL3, G-CSF, GM-CSF, SCF), $PDK1^{Hi}$ cells still retained a survival advantage (*Online Supplementary Figure S2*). These data suggest that overexpression of PDK1 promotes the survival of AML blasts.

To establish whether this association was causally related, we over-expressed PDK1 in the monocytic cell line, THP1. We found that THP1 cells ectopically expressing PDK1 displayed a 61% increase in survival compared to controls using 7AAD exclusion under reduced serum conditions (Figure 2B). Similarly, overexpression of PDK1 in normal $CD34^{+}$ cells also resulted in significantly improved cell survival (Figure 2C).

Together these data indicate that overexpression of PDK1 promotes the survival of myelomonocytic AML blasts.

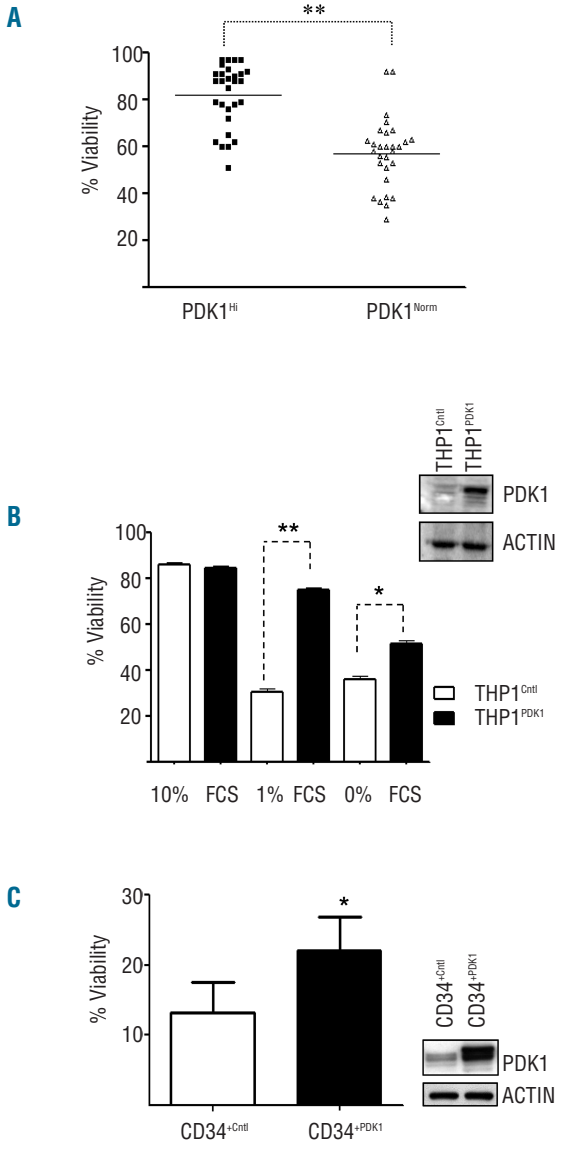

Figure 2. PDK1 overexpression promotes *in vitro* cell survival in AML blasts. (A) Viability of $PDK1^{Hi}$ AML blasts (n=28) compared with PDK1Norm AML blasts (n=29) scored by trypan blue at 48 h in cytokine-free medium ($P<0.001$). (B) Viability of THP1 cells over-expressing PDK1 (THP1PDK1) compared with vector control cells ($THP1^{Cntl}$) by 7AAD exclusion after 48 h of reduced serum culture; *$P<0.05$, **$P<0.001$. Top panel shows PDK1 expression in each line by Western blot. (C) Equivalent analysis of the effect of PDK1 over-expression (indicated by Western blot) on the survival of normal $CD34^{+}$ cells (0.5% FCS; n=4;*$P<0.05$ paired t-test).**

PDK1 promotes survival through PKC activation

To establish which of the downstream targets of PDK1 was responsible for mediating the pro-survival effect of PDK1, we carried out a survey of PDK1 target kinases including AKT, SGK, S6K, PKC, RSK and PAK. Of these RSK, PAK and S6K phosphorylation was not detected (*data not shown*) though we were able to detect phosphorylation of the S6K substrate, S6. In a sample of 20 myelomonocytic patients, no association between PDK1 overexpression and the phosphorylation of AKT, SGK1/2 or S6 was observed (Figure 3A); however, we did find that the level of PDK1 expression correlated directly with the phosphorylation of conventional and novel isoforms of PKC (Figure 3A and B).

These data imply that PDK1 expression is a limiting factor in PKC phosphorylation in this context and that the survival of AML blasts *in vitro* is related to their level of PKC phosphorylation. In support of this, we observed a close correlation between PKC phosphorylation and the *in vitro* survival of AML blasts (Figure 3C). Furthermore, inhibition of PKC using 2 different pan-isoform PKC inhibitors: Chelerythrine chloride (CC, peptide site competitive PKC inhibitor) and Bisindolylmaleimide 1 (BIM1, adenosine triphosphate site competitive PKC inhibitor) was able to abrogate the pro-survival effect of PDK1 overexpression in THP1 cells (Figure 3D and *Online Supplementary Figure S3A-C*). Since phospho-specific antibodies are not available for each PKC isoform, and membrane localization is key to activity, we analyzed PKC isoform activation by assessing their membrane localization in plasma membrane fractions derived from AML patients. This analysis showed a direct correlation between PDK1 expression and the membrane translocation of PKCα (Figure 3E and F).

Overall these data suggest that PDK1 promotes PKC activity in the context of myelomonocytic AML and that this contributes to their increased *in vitro* survival.

AML blasts are selectively sensitive to PDK1 inhibition

The above data suggested that targeting PDK1 could be effective in the treatment of myelomonocytic AML patients. To determine whether AML blasts over-expressing PDK1 were selectively sensitive to PDK1 inhibition, we assessed the efficacy of BX-795,[32] a pyrimidine derivative with high potency for PDK1 *in vitro* (IC50 10-30nM) using flow cytometry. We found that BX-795 was effective against PDK1^{Hi} AML blasts, but showed little toxicity against normal bone marrow CD34^{+} blasts (Figure 4A). In addition, PDK1^{Hi} AML blasts displayed twice the sensitivity to PDK1 inhibition compared to PDK1^{Norm} blasts

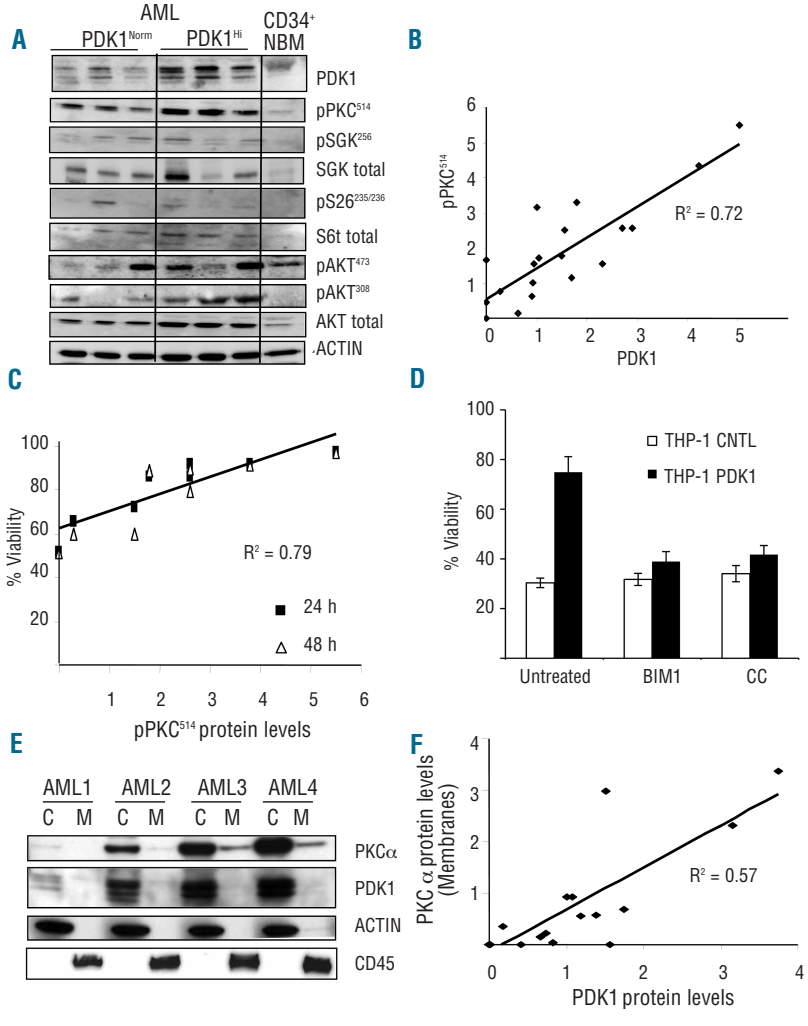

Figure 3. PDK1 expression correlates with PKC phosphorylation and pro-survival function in myelomonocytic AML. (A) Representative data (from 6 of 20 patients assayed) illustrating PDK1 target kinase phosphorylation in PDK1^{Norm} and PDK1^{Hi} patients. (B) Correlation of PDK1 and pPKC protein levels (n=20 patients). (C) pPKC levels in relation to cell viability at 24 h and 48 h (n=8 patients). pPKC units represent fold change relative to expression level in normal bone marrow (NBM) CD34^{+} cells (n=5). (D) Cell survival assay under serum deprived conditions (measured by MTS) of THP1PDK1 and THP1^{cntl} cells in the presence of Chelerythrine (CC, 0.3 μM) and Bisindolylmaleimide 1 (BIM1, 7.5 μM). (E) Representative Western blot of effect of PDK1 expression on the membrane localization of PKCα in primary AML samples (C: cytoplasmic; M: membrane). CD45 staining demonstrates purity of the membrane fractions (F) Summary data of the relationship between PDK1 expression and level of membrane-associated PKCα measured by Western blot of fractionated primary AML samples (n=15). Units represent fold change relative to expression levels in normal bone marrow (NBM) CD34^{+} cells (n=5).

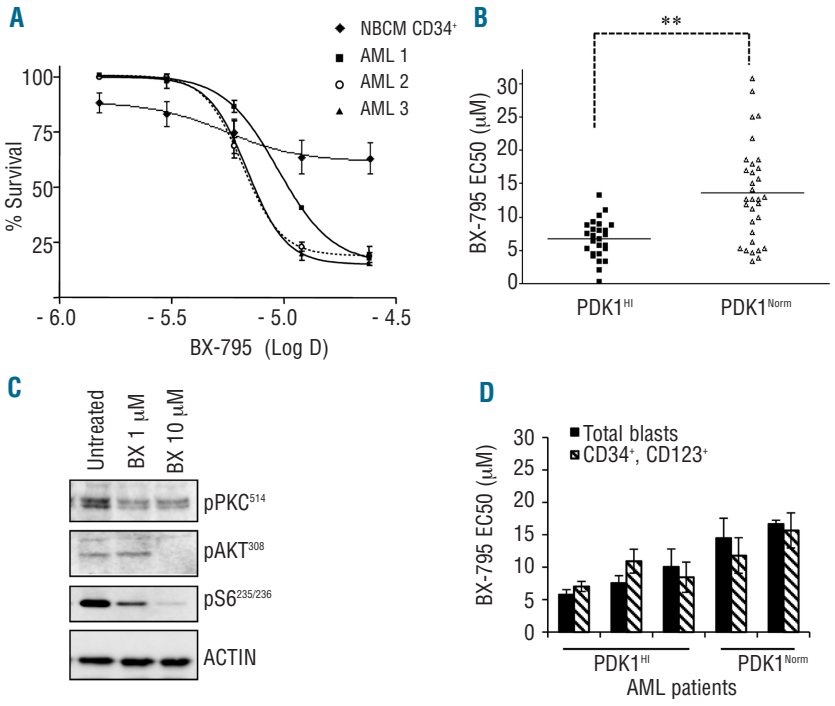

Figure 4. BX-795 induces cell death in AML blasts over-expressing PDK1. (A) Effect of BX-795 on the survival of normal bone marrow (NBM) CD34+ blasts (n=6) and representative PDK1Hi primary AML blasts. Survival is expressed as percentage viability compared with cells treated with vehicle alone. (B) Half maximal effective concentration (EC50) of BX-795 between PDK1Hi (n=26) and PDK1Norm (n=32) M4/M5 AML blasts (**P<0.001). (C) Western blot analysis of PDK1 target kinase phosphorylation following 60 min incubation with BX-795; data representative of 9 patient samples analyzed. (D) BX-795 EC50 levels in the total AML blast population compared with the CD34+, CD123+ subpopulation.

(PDK1Hi 6.4 μM±2.8 *vs.* PDK1Norm 13.6 μM±7.3) (Figure 4B). As predicted from previous studies[19,32] BX-795 was able to inhibit the phosphorylation of PKC and S6 in these patients (Figure 4C). Inhibition of AKT phosphorylation was also observed, but only at high dose.

To establish whether PDK1 inhibition could also effectively target leukemic sub-populations, we examined whether CD34+/CD123+ leukemic cells were also sensitive to PDK1 inhibition. Across all patients surveyed, we found that CD34+/CD123+ leukemic cells were equally or more sensitive to BX-795 compared with CD34neg leukemic subpopulations (Figure 4D) suggesting that all subpopulations are sensitive to PDK1 inhibition.

Since the use of new agents in AML is normally combined with cytarabine (AraC), we examined the effectiveness of AraC and BX-795 when used in combination. We found moderate synergy between the two agents in cell lines and in most primary AML (*Online Supplementary Figure S4*).

Overall these data show that PDK1 inhibition is effective in AML, particularly where PDK1 is over-expressed and that PDK1 inhibitors could be used to augment the activity of existing regimens.

Discussion

PDK1 is a constitutively active kinase[12,13] and no gain of function mutations have yet been identified in human cancer. In this context, therefore, it is unsurprising that overexpression of PDK1 is frequently observed in a variety of human cancers.[20-23] We show here that PDK1 overexpression is frequently observed in myelomonocytic AML and that expression is uniformly expressed throughout the AML blast population (including putative LIC) implicating PDK1 overexpression in the pathogenesis of AML. In support of this, overexpression of PDK1 has been implicated in the development of prostate and pancreatic cancer *in vivo*[25,33-35] and has been shown to transform mouse mammary epithelial cells *in vitro*.[36] The mechanism of overexpression is unclear; it has been reported that Meis3 up-regulated PDK1 transcription;[37] however, in our microarray database of 205 AML patients we found no correlation between Meis3 and PDK1 mRNA expression which also correlated poorly with PDK1 protein levels (*data not shown*). This observation is in accord with our previous data that suggested that PDK1 overexpression in this context arises from a post-translation mechanism.[27] In terms of treatment outcome, our data show that overexpression of PDK1 in myelomonocytic AML was associated with poor treatment outcome which could be linked with the cytoprotective effect of PDK1 overexpression that we observed both in primary AML blasts and cell lines. This compliments several other studies where PDK1 is known to have a pro-survival role in cancer cells.[23,35,38] Although AKT is a substrate for PDK1, and is commonly associated with pro-survival signaling, we were unable to demonstrate a correlation between PDK1 expression and the phosphorylation of AKT, possibly because this phosphorylation is also dependent on the level of PIP3. Data employing a new highly selective PDK1 inhibitor (GSK2334470) suggest that only very low levels of active PDK1 are required to efficiently phosphorylate AKT;[39] hence AKT phosphorylation is not limited by PDK1 expression level and it is, therefore, unlikely that PDK1 overexpression is driven by the need to promote phosphorylation of AKT. In support of this, PTEN tumor suppressor or PIK3CA oncogene mutations strongly drive PDK1 activation but are associated with only minimal activation of AKT.[38] Of all the PDK1 substrates whose phosphorylation was detectable in AML blasts, we observed that only the phosphorylation of PKC isoenzymes was directly

related to PDK1 expression level. Further investigation showed PDK1 expression to be closely associated with increased activation of PKCα. Overexpression of PDK1 in mammary epithelial cells similarly promotes transformation through activation and stabilization of PKCα but not AKT.[40] PKCα has also been linked to poor survival in AML41 and has been associated with blocking apoptosis in myelomonocytic patients,[42] suggesting hyperactivation of this pathway is advantageous in promoting treatment resistance in these patients. These reports, together with our own data, strongly implicate PKCα in the *in vitro* survival advantage of AML blasts over-expressing PDK1, although it is possible that PDK1 overexpression may also contribute to the activation of other targets, such as AKT, that may provide cooperative pro-survival function.

In contrast to normal blasts, we found that $PDK1^{Hi}$ AML blasts were highly sensitive to PDK1 inhibition. Furthermore, $PDK1^{Hi}$ blasts also showed greater sensitivity to inhibition than corresponding $PDK1^{Norm}$ blasts, suggesting that overexpression marked a stronger dependency on PDK1 to maintain their viability. PDK1 overexpression may, therefore, be an example of 'oncogene addiction' (where cancer cells become dependent on one or several genes for maintenance of cell survival).[43] Other PDK1 targeting agents have shown selectivity for AML *versus* normal blasts[44] and BX compounds have also been shown to selectively promote the apoptosis of breast cancer cells compared with normal epithelial cells.[32] Our data also showed that phenotypic leukemic subpopulations ($CD34^+$ $CD123^+$) present in myelomonocytic AML were equally sensitive to PDK1 inhibition compared with the bulk leukemic population, indicating that this agent also has the potential to target subpopulations associated with LIC,[31] although further *in vivo* engraftment experiments would be required to prove this.

Targeting PDK1 holds the promise of inhibiting a variety of signaling pathways associated with leukemogenesis while retaining specificity for a single gene product that is functionally non-redundant. This situation contrasts with some of the complexities that have arisen from attempting to target PKC directly.[45] Our data also show that PDK1 inhibition decreased the phosphorylation of all detectable PDK1 targets and it is likely that this combined effect contributed to the efficacy of BX-795.

In summary, we have identified PDK1 targeting in myelomonocytic AML as a novel therapeutic strategy with the potential for good therapeutic index, targeting the entire leukemic clone (including putative LIC), and it is predicted to be a safe and effective adjunct to conventional treatment.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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